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PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**UTILITY APPLICATION AND APPLICATION FEE TRANSMITTAL**  
**(1.53(b))**

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Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

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For: IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE PROTEIN  
CONJUGATE USEFUL AS A VACCINE PRODUCED USING AN N-  
ACRYLOYLATED POLYSACCHARIDE

Enclosed are:

[X] 33 page(s) of specification, 1 page(s) of Abstract, 7 page(s) of claims

[X] 1 sheets of drawing [X] formal [ ] informal

[X] 6 page(s) of Declaration and Power of Attorney

[ ] Unsigned  
[X] Newly Executed  
[ ] Copy from prior application

[ ] Deletion of inventors including Signed Statement under 37 C.F.R. § 1.63(d)(2)

[ ] Incorporation by Reference: The entire disclosure of the prior application, from which a copy of the combined declaration and power of attorney is supplied herein, is considered as being part of the disclosure of the accompanying application and is incorporated herein by reference.

[ ] Microfiche Computer Program (Appendix)

[ ] \_\_\_\_\_ page(s) of Sequence Listing

[ ] computer readable disk containing Sequence Listing  
[ ] Statement under 37 C.F.R. § 1.821(f) that computer and paper copies of the Sequence Listing are the same

[X] Claim for Priority: Provisional U.S. Application No. 60/097,120 filed August 19, 1998.

- ☐ Certified copy of Priority Document(s)
- ☐ English translation documents
- ☐ Information Disclosure Statement
- ☐ Copy of \_\_\_\_ cited references
- ☐ Copy of PTO-1449 filed in parent application serial No. \_\_\_\_\_.
- ☐ Preliminary Amendment
- ☒ Return receipt postcard (MPEP 503)
- ☒ Assignment Papers (assignment cover sheet and assignment documents)
- ☒ A check in the amount of \$40.00 for recording the Assignment.
- ☐ Assignment papers filed in parent application Serial No. \_\_\_\_\_.
- ☐ Certification of chain of title pursuant to 37 C.F.R. § 3.73(b).
- ☒ This is a ☐ continuation ☐ divisional ☒ continuation-in-part (C-I-P) of prior application serial no. 60/097,120.
- ☐ Cancel in this application original claims \_\_\_\_\_ of the parent application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)
- ☐ A preliminary Amendment is enclosed. (Claims added by this Amendment have been properly numbered consecutively beginning with the number following the highest numbered original claim in the prior application.
- ☒ The status of the parent application is as follows:
- ☐ A Petition For Extension of Time and a Fee therefor has been or is being filed in the parent application to extend the term for action in the parent application until \_\_\_\_\_.
- ☐ A copy of the Petition for Extension of Time in the co-pending parent application is attached.
- ☒ No Petition For Extension of Time and Fee therefor are necessary in the co-pending parent application.
- ☐ Please abandon the parent application at a time while the parent application is pending or at a time when the petition for extension of time in that application is granted and while this application is pending has been granted a filing date, so as to make this application co-pending.
- ☐ Transfer the drawing(s) from the patent application to this application.
- ☒ Amend the specification by inserting before the first line the sentence:  
This is a ☐ continuation ☐ divisional ☒ continuation-in-part of co-pending application Serial No. 60/097,120 filed August 19, 1998.

## I. CALCULATION OF APPLICATION FEE (For Other Than A Small Entity)

	Number Filed		Number Extra	Rate	Basic Fee
Total Claims	82	-20=	62	x\$18.00	\$ 760.00
Independent Claims	4	- 3=	1	x\$78.00	\$ 78.00
Multiple Dependent Claims					
	[X] yes		Additional Fee =	\$260.00	\$ 260.00
	[ ] no		Add'l Fee =	NONE	

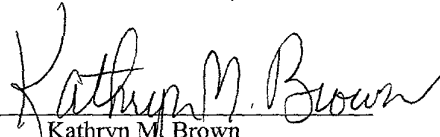
Total: \$2,214.00

- [ ] A statement claiming small entity status is attached or has been filed in the above-identified parent application and its benefit under 37 C.F.R. § 1.28(a) is hereby claimed. Reduced fees under 37 C.F.R. § 1.9(F) (50% of total) paid herewith \$ \_\_\_\_\_.
- [X] A check in the amount of \$2,214.00 in payment of the application filing fees is attached.
- [ ] Charge Fee(s) to Deposit Account No. 13-4500. Order No. \_\_\_\_\_. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.
- [X] The Assistant Commissioner is hereby authorized to charge any additional fees which may be required for filing this application, or credit any overpayment to Deposit Account No. 13-4500, Order No. 1758-4043US1. A DUPLICATE COPY OF THIS SHEET IS ATTACHED.

Respectfully submitted,

MORGAN &amp; FINNEGAN, L.L.P.

By:

  
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Michon et al Group Art Unit: To be assigned  
Serial No. : To be assigned Examiner: To be assigned  
Filed : August 18, 1999  
For : IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE  
PROTEIN CONJUGATE USEFUL AS A VACCINE PRODUCED USING  
AN N-ACRYLOYLATED POLYSACCHARIDE

EXPRESS MAIL CERTIFICATE

Express Mail Label No. EJ606944251US

Date of Deposit August 18, 1999

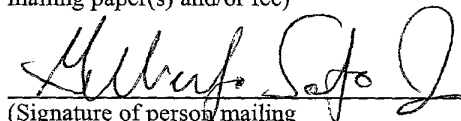
I hereby certify that the following attached paper(s) and/or fee

1. Utility Application and Application Fee Transmittal
2. Specification (33 pages)
3. Abstract (1 page)
4. Claims (7 pages, 82 claims)
5. Drawing (one sheet, Figure 1)
6. Declaration and Power of Attorney
7. Assignment Recordation Form Cover Sheet
8. Assignment
9. Check in the amount of \$2,214.00
10. Check in the amount of \$40.00
11. Return Receipt Postcard

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Gilberto Soto, Jr.

(Typed or printed name of person  
mailing paper(s) and/or fee)



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**IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE  
PROTEIN CONJUGATE USEFUL AS A VACCINE PRODUCED USING AN N-  
ACRYLOYLATED POLYSACCHARIDE**

**FIELD OF THE INVENTION**

The present invention relates to immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates and methods for producing the conjugates from bacteria, yeast, or cancer cells. The conjugates are useful as vaccines.

**BACKGROUND OF THE INVENTION**

Bacterial infections caused by gram-positive bacteria such as Streptococcus, Staphylococcus, Enterococcus, Bacillus, Corynebacterium, Listeria, Erysipelothrix, and Clostridium and by gram-negative bacteria such as Haemophilus, Shigella, *Vibrio cholerae*, Neisseria and certain types of *Escherichia coli* cause serious morbidity throughout the world. This, coupled with the emerging resistance shown by bacteria to antibiotics, indicates the need for the development of bacterial vaccines. For example, streptococci are a large and varied genus of gram-positive bacteria which have been ordered into several groups based on the antigenicity and structure of their cell wall polysaccharide (26,27). Two of these groups have been associated with serious human infections. The group A streptococci cause a variety of infectious disorders including "strep throat", rheumatic fever, streptococcal impetigo, and sepsis. Group B streptococci are important perinatal pathogens in the United States as well as developing countries (37).

Gram-negative bacteria are also a significant cause of disease. Until the recent development and use of polysaccharide-protein vaccines directed against *Haemophilus influenzae* type b bacteria (Hib), Hib bacterial infections were responsible for many cases of mental retardation in infants. *N. meningitidis* and *E. coli* K1 infections are responsible for neonatal meningitis. Strains of gram-negative bacteria, *E. coli*, have been linked to serious illness including death from eating meat tainted with *E. coli* strains.

The polysaccharides have been used to elicit antibody responses to a variety of gram-negative and gram-positive bacteria, when conjugated to another immunogenic molecule such as a polypeptide or protein. Conjugation of the

polysaccharide or oligosaccharide to the polypeptide converts the immune response to the polysaccharide or oligosaccharide which is typically T-cell independent to one which is T-cell dependent.

The prior art discloses both direct coupling and indirect coupling of polysaccharides to proteins to form conjugates (summarized in Ref. (11) and U.S. Patent No. 5,306,492). Conjugation methods have included diazo coupling, thioether bond, amidation, reductive amination and thiocarbamoyl for coupling a polysaccharide to a protein carrier.

Geyer et al., Med. Microbiol. Immunol., 165: 171-288 (1979) describes conjugates of certain *Klebsiella pneumoniae* capsular polysaccharide fragments to a nitrophenyl-ethylamine linker by reductive amination and attachment of the derivatized sugar using azo coupling.

U.S. Patent No. 4,057,685 by McIntire describes a *Escherichia coli* lipopolysaccharide with reduced toxicity covalently coupled to a protein antigen by reaction with haloacyl halide.

U.S. Patent No. 4,356,170 by Jennings et al. describes the production of polysaccharide-protein conjugates by reductive amination.

U.S. Patent No. 4,673,574, 4,761,283 and 4,808,700 by Anderson describes the production of immunogenic conjugates comprising the reductive amination product of an immunogenic capsular polysaccharide fragment derived from the capsular polymer of *Streptococcus pneumoniae* or *H. influenzae* containing a reducing end prepared by means such as oxidative cleavage with periodate or by hydrolyses of a glycosidic linkage, with a bacterial toxin or toxoid as a protein carrier.

U.S. Patent No. 4,459,286 by Hillman et al. describes the preparation of a polysaccharide-protein conjugate by activation of the *H. influenzae* type b polysaccharide with cyanogen bromide, derivatization of the activated polysaccharide with the spacer molecule, 6-aminocaproic acid, and the conjugation of the major outer membrane protein of *Neisseria meningitidis* with a water soluble carbodiimide to form an amido type of linkage to the protein through a complex variety of linkages from the 6-aminocaproic acid spacer to the polysaccharide.

U.S. Patent No. 4,965,338 by Gordon describes the production of a water-soluble covalent polysaccharide-diphtheria toxoid conjugate, wherein a pure *H.*

*influenzae* type b polysaccharide is activated with cyanogen bromide and immediately mixed with diphtheria toxoid which has been derivatized with an ADH spacer.

U.S. Patent No. 4,663,160 by Tsay et al. describes a detoxified polysaccharide from a gram-negative bacteria covalently coupled to a detoxified protein  
5 from the same species of gram-negative bacteria by means of a 4-12 carbon moiety.

U.S. Patent No. 4,619,828 by Gordon et al describes conjugates between polysaccharide molecules from pathogenic bacteria such as *Haemophilus influenzae* type B, *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Escherichia coli* and T cell dependent antigens such as diphtheria and tetanus toxoids.

U.S. Patent No. 4,711,779 by Porro et al describes glycoprotein conjugate  
10 vaccines having trivalent immunogenic activity comprising antigenic determinants from the capsular polysaccharides of a gram-positive bacteria, as well as either CRM<sub>197</sub>, tetanus toxoid, or pertussis toxin.

U.S. Patent No. 5,306,492 by Porro describes an oligosaccharide-carrier  
15 protein conjugate produced by reacting an oligosaccharide having a terminal reducing group with diaminomethane in the presence of pyridine borane such that reductive amination occurs, reacting the aminated oligosaccharide product with a molecule having two functional groups, and then reacting the activated oligosaccharide product with a carrier protein.

U.S. Patent No. 5,192,540 by Kuo et al describes a polysaccharide-protein  
20 conjugate comprising the reductive amination product of an oxidized polyribosyl-ribitol-phosphate polysaccharide fragment derived from the capsular polysaccharide of *Haemophilus influenzae* type b and the outer membrane protein of *Haemophilus influenzae* type b.

European publication No. EP 0747063 A2 describes a modified capsular  
25 polysaccharide containing multiple sialic acid derivatives and a heterobifunctional linker molecule linked to a carrier molecule. The linkers are used to N-alkylate up to about 5 sialic residues per polysaccharide. The remaining amino groups are then acylated with propionic or acetic anhydride.

More efficient, higher yielding and simpler means of obtaining purified  
30 immunogenic polysaccharide-protein conjugates for large-scale production of immunogenic polysaccharide-protein conjugate vaccines are desirable.

## SUMMARY OF THE INVENTION

The invention is an immunogenic  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-protein conjugate.

It is an object of this invention to provide a method for preparing  
5 immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates which provide advantages over currently employed methodologies. It is a further object of this invention to provide pharmaceutical compositions, vaccines and other immunological reagents derived from the immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates.

10 A method of preparing an immunogenic polysaccharide-protein conjugate is provided which comprises de-N-acetylation of a polysaccharide or an oligosaccharide by base or enzymatic hydrolysis followed by N-acryloylation of the N-deacetylated polysaccharide. The N-acryloylated polysaccharide is directly coupled to a carrier protein to form the immunogenic  $\beta$ -propionamido-linked polysaccharide-  
15 protein conjugate.

Capsular and cell surface polysaccharides can be extracted according to this invention from either bacterial, yeast, or mammalian cell supernatants or directly from bacterial, yeast or mammalian cells by hydrolysis of the base labile bond that connects the polysaccharide to other cellular components or by enzymatic hydrolysis.  
20 A percentage of the N-acetyl groups removed by hydrolysis from the polysaccharide are replaced by N-acryloyl groups, which in turn, are directly coupled to protein to form the conjugate of the present invention.

An aspect of the invention provides oligosaccharides and polysaccharides that are directly coupled at multiple sites to protein(s).

25 Another aspect of the invention is a method of immunizing a mammal against bacterial or yeast infections or cancer, which comprises administration to the mammal an effective amount of the vaccine of the invention for prevention against infection from a disease causing organism or cancer.

An aspect of the invention is a method of eliciting the production of  
30 antibodies in mammals using the  $\beta$ -propionamido-linked polysaccharide-protein conjugates that protect the mammals against infection or disease.



Another aspect of the invention is immunoglobulin and isolated antibody elicited in response to immunization using  $\beta$ -propionamido-linked polysaccharide-protein conjugates. Such immunoglobulin and isolated antibody are useful as a therapeutics and as diagnostic reagents.

5

#### **BRIEF DESCRIPTION OF THE DRAWING**

Figure 1. Schematic of the method of making the immunogenic  $\beta$ -propionamido-linked polysaccharide-proteinconjugates.

#### **DETAILED DESCRIPTION OF THE INVENTION**

661004-001000

The invention is a novel polysaccharide-protein conjugate and

10 oligosaccharide-protein conjugates useful as immunogens and vaccines against bacterial infections, yeast infections and as cancer therapeutics. Polysaccharides or oligosaccharides useful in forming immunogenic  $\beta$ -propionamido-linked polysaccharide-protein conjugates are derived from a source of polysaccharide or oligosaccharide which includes but is not limited to Gram (+) or Gram (-) bacteria,

15 yeast, cancer cells or cancerous tissues and the like in which the polysaccharide or oligosaccharide serves as a virulence factor for the cell in evading host defense mechanisms. The polysaccharide-protein conjugates of the present invention are formed by direct coupling of the N-acryloylated polysaccharide with a protein by a Michael-type addition of nucleophilic sites on proteins.

20 Polysaccharides or oligosaccharides may be obtained from a variety of sources including gram-negative, gram-positive bacteria, yeast, cancer cells or recombinant forms of each using base or enzymatic hydrolysis of the bond that attaches the polysaccharide or oligosaccharide to the cellular components. Polysaccharide or oligosaccharide may be extracted from the organism or cell by

25 contacting the organism or cell or a solution containing fragments of the organism or cell with an base or enzyme. Polysaccharide or oligosaccharide may then be recovered after basic or enzymatic hydrolysis by a variety of methods. Non-limiting examples of gram-positive bacteria and recombinant strains thereof for use according to this invention are Streptococci, Staphylococci, Enterococci, Bacillus,

30 Corynebacterium, Listeria, Erysipelothrix, and Clostridium. Specifically, the use of Streptococci is more preferred and the use of group B Streptococci types Ia, Ib, II, III, IV, V, and VIII is most preferred. Non-limiting examples of gram-negative bacteria

and recombinant strains thereof for use with this invention include *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Specifically, the use of *H. influenzae* type b, *N. meningitidis* types B, C, Y and W135, *E. coli* K1, and *E. coli* K92 are more preferred. Examples of yeast for use in the present invention include but are not limited to *Cryptococcus neoformans*. Examples of cancer cells or cancerous tissue for use in the present invention include but are not limited to small cell lung carcinoma, neuroblastomas, breast cancer, colon carcinoma, and the like.

A wide variety of conditions can be used for hydrolysis of the polysaccharide or oligosaccharide in either aqueous or organic solvent according to the invention by methods known in the art. The extent to which N-acetyl bonds of the carbohydrates are hydrolyzed can be controlled by the reaction conditions. In one embodiment, at least about 50% of the N-acetyl groups are removed by hydrolysis, preferably about 50% to about 100% are removed, more preferably about 90% or more of the native N-acetyl groups are removed. In a particular embodiment, about 95% or more of the N-acetyl groups are hydrolyzed from the polysaccharide by treatment with a hydrolysis reagent.

Capsular polysaccharides amenable to base extraction are those polysaccharides that lack any base-labile substituent that cannot be replaced, such as O-acetyl groups critical to immunogenicity. Other capsular polysaccharides amenable to base extraction are those lacking a phosphodiester bond and those lacking 4-linked uranic acid residues.

In a preferred embodiment for base hydrolysis, the CPS are extracted from group B Streptococci (GBS). In a most preferred embodiment the CPS are extracted from GBS types Ia, Ib, II, III, V and VIII.

In another preferred embodiment for base hydrolysis, the CPS are extracted from *S. pneumoniae*. In a more preferred embodiment for base hydrolysis the CPS are extracted from *S. pneumoniae* types III, IV and XIV.

In another preferred embodiment for base hydrolysis, the CPS are extracted from *Neisseria* or *Escherichia* bacteria. In a more preferred embodiment for base extraction, the CPS are extracted from *Neisseria meningitidis* types B, C, Y or W135, *Escherichia coli* K1 or *Escherichia coli* K92.

Polysaccharides amenable to enzymatic de-acetylation are those polysaccharides that lack any enzyme-labile substituent critical to immunogenicity in which the substituent cannot be replaced or substituted by an immunogenic moiety, these polysaccharides include but are not limited to GBS and the like.

5                    **A.      Preparation of the N-acryloylated polysaccharides**

**1.      Deacetylation of Polysaccharides**

**a).     Starting Materials**

Polysaccharide or oligosaccharide may be obtained using base hydrolysis or enzymatic hydrolysis from concentrated bacterial, yeast, mammalian  
10    cells or recombinant forms of these cells or from supernatants from homogenized cells or from conditioned medium using standard methods known in the art. The polysaccharide or oligosaccharide may be isolated and purified by standard methods known in the art. Isolated and purified polysaccharide or oligosaccharide from commercial sources may also be used as starting material.

15                    Methods for isolation of the polysaccharide depend on the particular polysaccharide being used. A common method is the use of ionic detergent to complex with a charged polysaccharide. The complex is precipitated and isolated. The complex is then dissolved in a solution of high ionic strength such as calcium chloride and the polysaccharide is then precipitated with ethanol

20                    The isolated and purified polysaccharides and oligosaccharides obtained for use in this invention preferably contain less than 1% nucleic acid and protein impurities for human use. Purities of 80-100% carbohydrate are often observed after purification due to the presence of inorganic salts.

**b).     Base Hydrolysis**

25                    To remove the N-acetyl groups the purified polysaccharides or oligosaccharides can be treated with bases. Non-limiting examples of bases which may be used according to this invention are NaOH, KOH, LiOH, NaHCO<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub>, KCN, Et<sub>3</sub>N, NH<sub>3</sub>, H<sub>2</sub>N<sub>2</sub>H<sub>2</sub>, NaH, NaOMe, NaOEt or KOtBu. Bases such as NaOH, KOH, LiOH, NaH, NaOMe or KOtBu are most effectively used in a range of  
30    0.5 N - 5.0 N. Bases such as NaHCO<sub>3</sub>, Na<sub>2</sub>CO<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub> and KCN can be used in concentrations as high as their solubilities permit. Organic bases such as Et<sub>3</sub>N can be used at medium to high (50-100%) concentrations as long as there is an agent such as

water or alcohol to effect the hydrolysis. Bases such as  $\text{NH}_3$  or  $\text{H}_2\text{N}_2\text{H}_2$  can be used at nearly any concentration including 100%. Solvents such as water, alcohols (preferably  $\text{C}_1\text{-C}_4$ ), dimethylsulfoxide, dimethylformamide or mixtures of these and other organic solvents can be used. Base solutions comprising water are most preferred.

The most effective pH range for removal of N-acetyl groups from the polysaccharide or oligosaccharide is from about 9 to about 14 with the optimal pH being around 12. The N-deacetylated polysaccharide thereafter is purified from residual reagents by ultrapurification using membranes or dialysis by standard methods known in the art.

#### c). **Enzymatic Hydrolysis**

The enzyme, N-deacetylase may be used to enzymatically removed N-acetyl groups from a polysaccharide or oligosaccharide. In one embodiment, an N-deacetylase enzyme useful in removal of N-acetyl residues from polysaccharides or oligosaccharides is described in Refs 47, 48 and 49. In enzymatic hydrolysis, the polysaccharide or oligosaccharide and deacetylase enzyme are mixed with an appropriate enzyme buffer system under appropriate pH and temperature conditions and allowed to react for a period sufficient for removal of N-acetyl groups. In one embodiment, polysaccharide and deacetylase enzyme are mixed with an appropriate enzyme buffer system, for example, 50 mM MES, 10 mM  $\text{MnCl}_2$ , pH 6.3 at  $37^\circ\text{C}$  for 60 minutes for formation of N-deacetylated polysaccharide. The reaction is stopped using an appropriate stopping solution for example 1 M monochloroacetic acid, 0.5 M NaOH, 2 M NaCl, or by dilution using an appropriate buffer solution.

#### 2. **N-Acryloylation of the Polysaccharide**

The alkaline or enzymatic hydrolysis of the polysaccharide or oligosaccharide results in the removal of N-acetyl groups from sialic acid and amino sugar residues of the polysaccharides or oligosaccharides. After hydrolysis, the polysaccharide or oligosaccharide is N-acryloylated to the extent desired by using a variety of acryloylating agents.

In one embodiment, the method comprises adding an acryloylating reagent to N-acryloylate an N-deacetylated polysaccharide or oligosaccharide. Examples of acryloylation reagents include but are not limited to acryloyl chloride,

acryloyl anhydride, acrylic acid and a dehydrating agent such as DCC,  $\text{CH}_2\text{CHCOCN}$  the like, used in excess at a concentration of about 1 M. In a method of N-acryloylation of an N-deacetylated polysaccharide, the pH is adjusted and maintained at about 9 to about 11, preferably about pH 10 during the reaction. The temperature during reaction is about 2°C to about 8°C, preferably about 4°C. The reaction is carried out over a period of about 1 hour. The resulting N-acryloylated polysaccharide or N-acryloylated oligosaccharide is at least about 95% acryloylated or greater.

**B. Preparation of  $\beta$ -propionamido-linked polysaccharide-protein conjugates**

The polysaccharide or oligosaccharide of this invention may be used to elicit antibody responses to a variety of gram-negative and gram-positive bacteria, yeast and cancers in an individual when conjugated to another immunogenic molecule such as a polypeptide or protein. Conjugation of the polysaccharide or oligosaccharide to the polypeptide converts the immune response to the polysaccharide or oligosaccharide which is typically T-cell independent to one which is T-cell dependent. Accordingly, the size of the polypeptide is preferably one which is sufficient to cause the conversion of the response from T-cell independent to T-cell dependent. It may be useful to use smaller polypeptides for the purpose of providing a second immunogen. The size of the protein carrier is typically from about 50,000 to about 500,000 M.W.

Preferred carrier proteins include, but are not limited to, tetanus toxoid, diphtheria toxoid, cholera toxin subunit B, *Neisseria meningitidis* outer membrane proteins, pneumolysin, C- $\beta$  protein from group B Streptococcus, non-IgA binding C- $\beta$  protein from group B Streptococcus, *Pseudomonas aeruginosa* toxoid, pertussis toxoid, synthetic protein containing lysine or cysteine residues, and the like. The carrier protein may be a native protein, a chemically modified protein, a detoxified protein or a recombinant protein. Conjugate molecules prepared according to this invention, with respect to the protein component, may be monomers, dimers, trimers and more highly cross-linked molecules.

This invention provides the ability to produce conjugate molecules wherein the protein is linked to the polysaccharide or oligosaccharide through one or more sites on the polysaccharide or oligosaccharide. The size of the polysaccharide or

oligosaccharide may vary greatly. One or a multiplicity of polysaccharides or oligosaccharides may cross-link with one or a multiplicity of protein. The conjugates of the present invention are preferably lattice structures. The points of attachment are between lysine or cysteine residues of the protein and the N-acryloyl groups of the polysaccharide or oligosaccharide.

In one method of forming a immunogenic polysaccharide-protein conjugate, an isolated polysaccharide (glycosaminoglycan) containing free amino groups or N-acyl groups (e.g. N-acetyl groups) in the sugar residues that constitute its repeating unit, is first treated hydrolyzed using base or enzyme to remove part or all of its N-acyl groups. The free amino groups are then N-acylated with an N-acryloylating reagent to form the N-acryloylated polysaccharide described above. The N-acryloylated polysaccharide is then directly coupled to protein under optimum conditions of pH, temperature and time to form an immunogenic  $\beta$ -propiionamido-linked polysaccharide-protein conjugate.

In one embodiment, the method of conjugation is conducted at a pH above 9.0, preferably a pH of about 9.0 to about 10.0 for optimal reactivity of  $\epsilon$ -free amino groups of lysine residues on the protein. In another embodiment, the method of conjugation is conducted at a neutral pH of about 7.0 for optimal reactivity of thiol (SH) groups of cysteine residues of the protein. The selection of pH for conducting the method of conjugation may be based on the number of reactive groups in a particular carrier protein. For example, a method using a protein composed of more reactive lysine residues as compared to cysteine residues is preferably conducted at a basic pH. A method of conjugation using a protein composed of more reactive cysteine residues as compared to lysine residues is preferably conducted at about a neutral pH.

The conjugation reaction may be conducted in buffered reagents including but not limited to a buffered reagent including carbonate/bicarbonate, borate buffer, phosphate and the like. The temperature of the conjugation reaction is at least about 25 °C, preferably about 37 °C, for a period of preferably about 24 hours. The key reaction involves a 1,4-conjugate addition (Michael-type addition) of nucleophilic cysteine thiol groups or lysine  $\epsilon$ -NH<sub>2</sub> groups on proteins with N-acryloylated sugar residues as described by Romanowska et al (46) which are present in the repeating-

unit of the polysaccharide as shown in Figure 1. The resulting  $\beta$ -propionamido-linked polysaccharide-protein conjugate has a polysaccharide to protein ratio of about 0.1 to about 0.6.

The glycosyl residues of the polysaccharide having N-acyl groups  
5 amenable to direct conjugation with cysteine and/or lysine residues on protein include, but are not limited to, glucosamine, galactosamine, mannosamine, fucosamine, sialic acids and the like. The polysaccharide may be derived from natural sources such as bacteria, yeast or cancer cells or from synthetic sources. Synthetic sources include chemical synthesis, enzymatic synthesis and chemoenzymatic synthesis. The  
10 synthesis may be *de novo* synthesis or the modification of natural carbohydrates. Naturally isolated carbohydrates can be modified by altering functional groups on carbohydrate residues or by the addition or removal of carbohydrate residues.

The polysaccharide or oligosaccharide for use in preparing the  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-  
15 protein conjugates of the present invention may vary in size for conjugation with a carrier protein. As defined herein, an oligosaccharide for use in the present invention comprises at least 10 sugar residues and preferably from 10 to about 50 sugar residues. A polysaccharide, as defined herein, is greater than 50 sugar residues and may be as large as about 600 or greater residues. In some cases, large constructs are desirable for  
20 enhancement of immunogenicity. The methods of this invention provide for the use of very large polysaccharides because many reactive sites can be introduced into a single polysaccharide. Another advantage of this method over the prior art is that the polysaccharide or oligosaccharide is not altered at a charged functional group which often interact with/or form part of the epitope crucial for immunity.

### **C. Vaccines**

This invention is also directed to vaccine preparations. According to this invention, the isolated  $\beta$ -propionamido-linked polysaccharide-protein conjugates described above may be used as an antigen to generate antibodies that are reactive  
5 against the polysaccharide or oligosaccharide and hence reactive against the organism or cell from which the polysaccharide or oligosaccharide was isolated. The vaccines of the present invention may be a combination or multi component vaccine further comprising in combination with the  $\beta$ -propionamido-linked polysaccharide-protein conjugate other components, including but not limited to Diphtheria-Tetanus-Pertusis  
10 (DTP), Tetanus-Diphtheria (Td), DTaP, a DTaP-Hib vaccine, a DTaP-IPV-Hib vaccine, and the like and combinations thereof, to provide a multifunctional vaccine useful in immunizing against a variety of diseases causing organisms or disease causing cells.

The vaccines of this invention may provide active or passive immunity.  
15 Vaccines for providing active immunity comprise an isolated and purified N-acryloylated polysaccharide or oligosaccharide conjugated to at least one antigenic peptide.

### **D. Pharmaceutical compositions**

The pharmaceutical compositions of this invention may comprise at  
20 least one polysaccharide-protein conjugate and pharmacologically acceptable carriers such as saline, dextrose, glycerol, ethanol or the like. In another embodiment the pharmaceutical composition comprises another immunogenic moiety, such as a peptide, or compositions comprising antibodies elicited by one of the CPS of this invention. The composition may also comprise adjuvants to enhance the  
25 immunological response of the recipient. Such adjuvants may be aluminum based such as alum or long chain alkyl adjuvants such as stearyl tyrosine (see U.S. Serial No. 583,372, filed 9/17/90; European Patent, EP 0 549 617 B1; Moloney et al. U.S. Patent No. 4,258,029), muramyl dipeptide (MDP) or derivative thereof, monophosphoryl lipid A (MPL), saponin (Quil-A) and the like. See also Jennings, et al. U.S Patent No.  
30 5,683,699 and Paoletti, et al. J. Infectious Diseases 1997; 175:1237-9. The pharmaceutical composition may further comprise one or more additional immunogens including but not limited to Diphtheria-Tetanus-Pertusis (DTP), Tetanus-



Diphtheria (Td), DTaP, DTaP-Hib, DTaP-IPV-Hib, and the like and combinations thereof. These pharmaceutical compositions are particularly useful as vaccines.

For eliciting passive immunity, the pharmaceutical composition may be comprised of polyclonal antibodies, or monoclonal antibodies, their derivatives or  
5 fragments thereof and recombinant forms thereof. The amount of antibody, fragment or derivative will be a therapeutically or prophylactically effective amount as determined by standard clinical techniques.

The pharmaceutical preparations of this invention may be introduced to an individual by methods known to be effective in the art. Intradermal,  
10 intraperitoneal, intravenous, subcutaneous, intramuscular, oral and intranasal are among, but not the only, routes of introduction.

The compositions of the invention may comprise standard carriers, buffers or preservatives known to those in the art which are suitable for vaccines including, but not limited to, any suitable pharmaceutically acceptable carrier, such as  
15 physiological saline or other injectable liquids. Additives customary in vaccines may also be present, for example stabilizers such as lactose or sorbitol and adjuvants to enhance the immunogenic response such as aluminum phosphate, hydroxide, or sulphate and stearyl tyrosine. The vaccines produced according to this invention may also be used as components of multivalent vaccines which elicit an immune response  
20 against a plurality of infectious agents.

Vaccines of the present invention are administered in amounts sufficient to elicit production of antibodies as part of an immunogenic response. The vaccine can be used parenterally to produce IgG and IgM antibodies or it can be delivered to the mucosal membranes to elicit IgA antibodies on the surface of tissues.  
25 Dosages may be adjusted based on the size, weight or age of the individual receiving the vaccine. The antibody response in an individual can be monitored by assaying for antibody titer or bactericidal activity and boosted if necessary to enhance the response. Typically, a single dose for an infant is about 10 µg of conjugate vaccine per dose or about 0.5 µg-20 µg/kilogram. Adults receive a dose of about 0.5 µg-20 µg/kilogram  
30 of the conjugate vaccine. For the CPS-protein conjugate vaccine, a typical dose is about 25 µg of each individual CPS per dose. That is, a vaccine against group B streptococcus may comprise 25 µg of each of the CPS form each of the nine serotypes.

### **E. Antibodies**

Antibodies directed against the polysaccharide may be generated by any of the techniques that are well known in the art. According to one approach, the antibodies may be generated by administering an isolated immunogenic  $\beta$ -  
5 propionamido-linked polysaccharide-protein conjugate into a host animal. The host animal may be, but is not limited to, rat, mouse, rabbit, non-human primate, or a human. Preferably, the host is human. In one embodiment, immunological responses may be increased by the use of adjuvants which are known in the art

Monoclonal antibodies directed against the polysaccharide may also be  
10 prepared by any of the techniques that are well known in the art. According to one method, cultures of hybridoma cell lines are used (Kohler and Milstein (1975) *Nature* 256:495-497). Monoclonal antibodies directed against the polysaccharide may be human monoclonal antibodies, chimeric monoclonal antibodies or humanized monoclonal antibodies made by any of the techniques that are well known in the art.  
15 According to one approach, chimeric monoclonal antibodies may be generated that have a non-human (e.g. mouse) antigen-binding domain combined with a human constant region. (Takeda et al. (1985) *Nature* 314:452). Humanized antibodies can be generated according to the procedures of Queen et al., U.S. Patent No. 5,585,089 and U.S. Patent No. 5,530,101. Single chain antibody may be constructed by methods  
20 known in the art (U.S. Patent No. 4,946,778; Davis, G.T. et al 1991 Biotechnology 9:165-169; Pluckthun, A. 1990 Nature 347:497-498). Constant region domains of the antibody may be modified by procedures known in the art (WO 89/07142)

Antibodies directed against the polysaccharide or oligosaccharide may be purified by any of the techniques that are well known in the art including, but not  
25 limited to immunoabsorption or immunoaffinity chromatography, or other chromatographic methods (e.g. HPLC). Antibodies may also be purified as immunoglobulin fractions from serum, plasma or cell culture medium.

Antibody molecules of this invention may be intact immunoglobulin molecules, substantially intact immunoglobulin molecules, or those portions of an  
30 immunoglobulin molecule, for example Fab fragments, that contain the antigen binding site. The antibody molecules may be of any class including IgG, IgM, and IgA.

Fragments of antibodies directed against the CPS may be generated by any of the techniques that are well known in the art. (Campbell (1985) Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 13, Burdon, et al. (eds.), Elsevier Science Publishers, Amsterdam).

5           The antibody or antigen or antigen binding fragment thereof is useful as a therapeutic in providing passive protection against diseases caused by Gram (+), Gram (-) bacteria or yeasts. The antibody or antigen binding fragment thereof are also useful as a diagnostic reagent in standard immunoassays for the detection and/or identification of bacteria, yeast or cancer cells. The antibody may be supplied in kit  
10   form alone or with standard reagents for immunoassays.

          In another embodiment of this invention, antibodies directed against the polysaccharide or oligosaccharide of this invention may be used as a pharmaceutical preparation in a therapeutic or prophylactic application in order to confer passive immunity from a host individual to another individual (i.e., to augment an individual's  
15   immune response against gram-negative or gram-positive bacteria or yeast or to provide a response in immuno-compromised or immuno-depleted individuals including AIDS patients). Passive transfer of antibodies is known in the art and may be accomplished by any of the known methods. According to one method, antibodies directed against the conjugates thereof of this invention are generated in an  
20   immunocompetent host ("donor") animal, harvested from the host animal, and transfused into a recipient individual. For example, a human donor may be used to generate antibodies reactive against the polysaccharide-protein conjugate of this invention. The antibodies may then be administered in therapeutically or prophylactically effective amounts to a human recipient in need of treatment, thereby  
25   conferring resistance in the recipient against bacteria which are bound by antibodies elicited by the polysaccharide component. (See Grossman, M. and Cohen, S. N., in "Basic and Clinical Immunology", 7th Ed., (Stites, D. P. and Terr, A. T. eds., Appleton & Lange 1991) Chapter 58 "Immunization".)

          In certain cases the polysaccharide used with this invention may induce  
30   antibody which is cross-reactive with other pathogenic organisms and thus have ability in protecting against infection by these other bacteria.

#### **F. Diagnostic kits**

In another embodiment, the CPS of this invention or derivatives or fragments thereof may be provided in diagnostic kits to indicate the presence of antibodies directed against bacteria, yeast or cancer cells. The presence of such antibodies can indicate prior exposure to the pathogen, and predict individuals who may be resistant to infection. The diagnostic kit may comprise at least one of the CPS of this invention or derivatives or fragments thereof, alone or conjugated to protein, and suitable reagents for the detection of an antibody reaction when the modified CPS or derivatives or fragments are mixed with a sample that contains antibody directed against gram-negative, gram-positive bacteria, yeast or cancer cells or cancer tissue. An antibody reaction may be identified by any of the methods described in the art, including but not limited to an ELISA assay. Such knowledge is important, and can avoid unnecessary vaccination.

Alternatively, the diagnostic kit may further comprise a solid support or magnetic bead or plastic matrix and at least one of the CPS of this invention or derivatives or fragments thereof.

In some cases, it may be preferred that the CPS or derivatives or fragments are labeled. Labeling agents are well-known in the art. For example, labeling agents include but are not limited to radioactivity, chemiluminescence, bioluminescence, luminescence, or other identifying "tags" for convenient analysis. Body fluids or tissues samples (e.g. blood, serum, saliva) may be collected and purified and applied to the diagnostic kit. The CPS, derivatives or fragments may be purified or non-purified and may be composed of a cocktail of molecules.

Solid matrices are known in the art and are available, and include, but are not limited to polystyrene, polyethylene, polypropylene, polycarbonate, or any solid plastic material in the shape of test tubes, beads, microparticles, dip-sticks, plates or the like. Additionally matrices include, but are not limited to membranes, 96-well micro titer plates, test tubes and Eppendorf tubes. In general such matrices comprise any surface wherein a ligand-binding agent can be attached or a surface which itself provides a ligand attachment site.

All publications, patents and articles referred to herein are expressly incorporated herein *in toto* by reference thereto. The following examples are

presented to illustrate the present invention but are in no way to be construed as limitations on the scope of the invention. It will be recognized by those skilled in the art that numerous changes and substitutions may be made without departing from the spirit and purview of the invention.

5

## EXAMPLE 1

### Preparation of $\beta$ -Propionamido-Linked Polysaccharide-Protein Carrier Conjugates

The following non-limiting examples describe the preparation of a series of clinically relevant polysaccharide-protein conjugates for vaccines against  
10 *Streptococcus pneumoniae* (type 14), Group B *Streptococcus* (GBS) type III and type II, and *E.coli* K1. All of the above polysaccharides used in this example are glycosaminoglycans that contain N-acetyl groups in one or more of the glycosyl residues that are constituents of their structural repeating-units.

#### A. Depolymerization of type 14 pneumococcal polysaccharide

15 To increase its solubility the polysaccharide was first partially depolymerized by sonication. 200 mg of *Pneumococcal polysaccharide* type 14 (Lot NO 2020510, American Type Culture Collection) was dissolved in 20 ml of PBS and sonicated for 4 hours at 0°C with a Branson Sonifier Model 450. The resulting polysaccharide was dialyzed and lyophilized and then sized through a superdex 200  
20 column equilibrated with phosphate buffered saline (PBS). Peak fractions were pooled and then dialyzed against d.i. water with Spectra/Por® Membrane MWCO:3,500. A yield of 157.5mg solid was obtained after lyophilization. The sonicated polysaccharide had an average molecular weight of about 50,000 as measured by SEC-MALLS with the miniDAWN (Wyatt Technology Corp., Santa Barbara, CA).

#### 25 B. De-N-Acetylation of type 14 pneumococcal polysaccharide

100 mg of sized type 14 pneumococcal polysaccharide was dissolved in 10 ml of 2N NaOH and then 10 mg of NaBH<sub>4</sub> was added to the reaction mixture. This mixture was heated at 100 °C for one hour and then cooled to room temperature. The N-deacetylated component was dialyzed against d.i. water with a Spectra/Por ®  
30 Membrane Membrane MWCO:3,500 and lyophilized to give 84 mg of white solid. The N-deacetylated polysaccharide was analysed by H<sup>1</sup>-NMR at 500 MHz and was found to contain less than 5 percent residual N-acetyl groups.

### **C. N-Acryloylation of the N-deacetylated type 14 Pneumococcal polysaccharide**

84 mg of N-deacetylated type 14 Pneumococcal polysaccharide was dissolved in 4.2 ml of d.i. water. The solution, in an ice bath, was adjusted to pH 10 with 2 N NaOH. Then 420  $\mu$ l of 1:1 v/v acryloyl chloride : dioxane was added and adjusted to pH 11 with 2 N NaOH. The reaction was allowed to stand for an additional hour at pH 11 to ensure the complete hydrolysis of esters which may have formed as a result of O-acylation. The solution was dialyzed and lyophilized to give 42 mg of dry powder. After analysis by 500 MHz  $H^1$ -NMR the polysaccharide was found to be over 95 percent N-acryloylated.

### **D. Coupling of the type 14 N-acryloylated pneumococcal polysaccharide to tetanus toxoid monomer**

22 mg of the type 14 N-acryloylated pneumococcal polysaccharide was dissolved in 1.1 ml of Carbonate/Bicarbonate pH 9.5 buffer. Tetanus toxoid monomer 22 mg was added to the reaction mixture. The reaction mixture was incubated overnight at 37 °C. The progress of the conjugation was analyzed with a Biologic system (Bio-Rad) equipped with a superose 12 column. Conjugation of polysaccharide to tetanus toxoid was indicated by the progressive increase in a peak, monitored by measurement of UV absorbance at 280 nm, eluting in the void volume of the column. After conjugation was complete, the solution was neutralized to pH 7 with 0.1N HCl and then dialyzed against PBS. The conjugate was purified by passage over a 1.6x60cm column of Superdex 200 PG (Pharmacia) and eluted with PBS containing 0.01% thimerosal. Fractions corresponding to the void-volume peak were pooled. Carbohydrate and protein content in the conjugate were estimated by the phenol-sulfuric assay of Dubois et al. (51) and the Coomassie assay of Bradford (9).

Similar methods were used for GBS type II, type III as well as for the *E. coli* K1 and meningococcal C polysaccharides. The reaction conditions for each of these polysaccharides are tabulated below.

**TABLE 1**

**E. De-N-Acetylation of GBS Type II and Type III Polysaccharide**

	PS size* (kD)	PS in mg	NaOH	NaBH <sub>4</sub>	Temp.	Reaction time	yield
GBSP II	250	63 mg	6 mL	12 mg	110 °C	6 h	63 mg
GBSP III	110	50 mg	5 mL	10 mg	110 °C	6 h	55 mg

5 \*Determined by SEC-MALLS

**TABLE 2**

**N-Acryloylation of GBS Type II and Type III polysaccharide**

	PS amount in mg	d.i. water	1:1 v/v acryloyl chloride:dioxane	yield
GBSP II	60	3 ml	300 µl	60 mg
GBSP III	55	2.75 mL	275 µl	55 mg

**TABLE 3**

**Coupling of the GBS II and GBS III polysaccharide to Tetanus Toxoid Monomer**

	PS in mg	TT in mg	Carbo/Bicarb buffer pH 9.5	temperature	Incubation time
GBSP II	10	10	0.5 mL	37 °C	overnight
GBSP III	10.52	9.52	0.5 mL	37 °C	overnight

15 **F. De-N-Acetylation of K1 polysaccharide**

300 mg of K1 PS was dissolved in 15 mL of 2.0 N NaOH solution to which 150 mg of sodium borohydride was added. The solution was heated at 110 °C for 6 hours, cooled down to room temperature and diluted with a 20-fold volume of dionized water. After diafiltration through an Amicon YM3 membrane with deionized water, the solution was lyophilized yielding 255 mg of N-deacetylated K1 PS. H<sup>1</sup>-  
20 NMR at 500 MHz confirmed that complete N-deacetylation occurred.

**G. N-Acryloylation of K1 polysaccharide**

To a 10 mL deionized water solution containing 250 mg of de-N-acetylated K1 PS, cooled in an ice bath, was added dropwise acryloyl chloride

(Aldrich, Milwaukee, WI) solution, prepared by combining 1 mL of acryloyl chloride with a 1 mL of dioxane. The pH of the solution was maintained between 7.0 and 10.5 by the addition of 2 N sodium hydroxide solution. After completion of the addition, the pH was raised to 13 and maintained between 12.9 to 13.1 for 1 hour at room temperature. The pH of the solution was adjusted to 9.5 by the dropwise addition of 1 N HCL. The solution was diafiltrated with an Amicon YM3 membrane in a stircell with deionized water. The retentate was lyophilized to dryness, and the material (N-Acryloyl K1 PS) was stored at in a desiccator in a -20 C freezer. H-NMR at 500 MHz indicated that complete N-acryloylation took place during the reaction.

#### **H. $\beta$ -Propionamido-Linked K1-rPorB conjugate (K1-rPorB I)**

A solution containing 8.4 mg of N-Acryloyl K1 PS and 4.0 mg of recombinant *Neisseria meningitidis* PorB in 0.3 mL of 0.2 M borate, 0.05% Zwittergen™ 3,14 (Boehringer Mannheim) pH 9.5 was incubated at 37° C for 3 days. The conjugate was purified by size exclusion chromatography through a Superdex 200 preparative grade column, and eluted with PBS containing 0.01% thimerosal. The fractions of uv-280 nm active signal eluting at or close to the void volume of the column were pooled and stored in the refrigerator. The conjugate was analysed for sialic acid and protein content by the resorcinol and Coomassie protein assays respectively.

#### **I. Preparation of Thiolated rPorB**

To one ml of rPorB porin solution at a conc of 10 mg/ml in 0.25 M HEPES buffer of pH 8.5 containing 0.25 M sodium chloride and 0.05% zwittergent 3-14 was added 0.2 ml of 0.05 M N-succinimidyl 3-[2-pyridyldithio]propionate solution. The solution was mixed well and allowed to sit at RT for one hour. To the solution was added 0.06 ml of 1 M dithiothreitol solution in the same buffer. The solution was again mixed well and allowed to sit at RT for an additional two hours. The solution was diluted with 1.3 ml of 0.25 M HEPES buffer of pH 7.0 containing 0.25 M sodium chloride and 0.05% zwittergent 3-14 and loaded onto a Pharmacia PD-10 desalting column which had been pre-equilibrated with the same buffer. The column was eluted with the same buffer, and eluate was collected and concentrated with an



Amicon Centricon 30 concentrator at 5,000 RPM for one hour. The retentate was collected and the protein concentration determined.

#### **H.. Preparation of N-Acryloylated K1-S-rPorB Conjugate (K1-S-PorB)**

5

To 0.17 ml of thiolated rPorB solution at a concentration of 25 mg/ml from above was added 9 mg of N-acryloylated K1 polysaccharide. The solution was mixed well and incubated in an oven of 37° C for 18 hours. The solution was purified through a Superdex 200 column (Pharmacia) with PBS as eluent. UV-280-nm-active  
10 fractions eluted at or close to the void volume of the column were combined. Analyses showed that the conjugate contained 25 ug/ml of polysaccharide and 188 ug/ml of protein.

#### **I. Preparation of N-Acryloylated GCMP-S-rPorB Conjugate (GCMP-S-rPorB)**

15

Likewise, N-acryloylated GCMP-S-rPorB was prepared in a procedure comparable to the one described above for N-acryloylated K1-S-rPorB conjugate and found to contain 43 ug/ml of polysaccharide and 200 ug/ml of protein.

20

**TABLE 4**  
**Analytical Data for the Conjugates Described Above**

	Protein Conc. μg/mL	PS Conc. μg/mL	Percent PS in conjugate
Pn14-TT (3)	547	293 (1)	35
GBSII-TT(3)	377	160 (2)	30
GBSIII-TT (3)	365	115 (2)	24
K1-rPorB I (3)	147	17 (2)	10
K1-rPorB II (4)	406	41 (2)	9
K1-S-rPorB (3)	188	25 (2)	12
GCMP-S-rPorB (3)	200	43 (2)	18

5

- (1) Total carbohydrate Dubois assay
- (2) resorcinol sialic assay
- (3) Prepared by direct coupling of the N-Acryloylated polysaccharide and the corresponding carrier protein
- 10 (4) Control conjugate prepared by reductive amination of a periodate-oxidized N-Acryloylated K1 PS with rPorB

## EXAMPLE 2

15

### **Immunogenicity and Potency of the β-Propionamido-Linked Polysaccharide-Protein Carrier Conjugates**

#### **Preclinical evaluation of the conjugates in mice**

- Immunoassays: Serum antibody to each polysaccharide conjugate was
- 20 measured by ELISA. The human serum albumin (HSA) (Sigma, St Louis, MO) conjugates used for ELISA assays were prepared by reductive amination. The oxidized polysaccharides were added to HSA followed by reductive amination with NaCNBH<sub>3</sub>. The conjugates were isolated by gel filtration chromatography, and stored freeze-dried at -70 °C. PS-specific antibody titers were determined by an ELISA as follows.
- 25 Polystyrene, 96-well, flat-bottom microtiter plates (NUNC Polysorb) (Nunc, Naperville, IL) were coated with PS-HSA conjugates in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.5 ) at 0.25 μg/well (100μL/ well) by incubating for 1 hour at 37 °C, followed by a PBS-Tween (0.05% v/v Tween 20 in PBS) wash (5
- 30 times). All subsequent incubations were conducted at room temperature. PBS-Tween was used for all required washes. The coated plates were then blocked with PBS-BSA

(0.5% w/v bovine serum albumin in PBS) for IgG ELISAs or 0.1% w/v Carnation nonfat dry milk for IgM ELISAs at 0.15 mL / well for 1 hour, followed by a wash. Sera were diluted 2-fold, in duplicate, in the plate at 100  $\mu$ L/ well and incubated for 1 hour, followed by a wash. Antibody conjugate (peroxidase-labelled goat anti-mouse (Kirkegaard & Perry Lab, Gaithersburg, MD) was added at 100  $\mu$ L/ well and incubated for 30 minutes, followed by a wash. A 1:1 dye and substrate solution (Kirkegaard & Perry TMB) and peroxide was added at 0.05mL/ well and incubated for 10 minutes. The peroxidase reaction was then stopped with 1 M  $H_3PO_4$  at 0.05 mL/ well, and the plate was read on a Molecular Devices Emax microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 450 nm, using 650 nm as a reference wavelength. Background absorbances were determined in several no-serum control wells and averaged for each plate. For each serum dilution, the average background absorbance was subtracted, and then duplicate serum absorbance values were averaged. A modified Scatchard plot was used for the subsequent data analysis, where the absorbance (y-axis) was plotted against the absorbance times the reciprocal dilution (x-axis) (ref). Under conditions allowing equilibrium and antibody excess, a straight line was obtained for each serum dilution series; this line was extrapolated to the x-axis for the determination of an antibody titer. A positive control serum, with a previously determined antibody titer, was used on each plate in order to provide a reference to which all sera were standardized, minimizing plate to plate and day to day variations. The results of these assays are shown in Tables 5, 6 and 7.

Opsonophagocytic assays (OP) : The opsonic activity of mice antisera to the Streptococcal B (GBS) and Pneumococcal conjugates was tested in an *in vitro* opsonophagocytic killing assay using the human promyelocytic leukemia HL-60 cell line (ATCC No. CCL 240). Briefly, 200 cfu of GBS type III strain M781 cells or pneumococcal type 14 strain were mixed in equal volume with serum antibodies and incubated under shaking 15 minutes at 35  $^{\circ}C$  in a 5%  $CO_2$  incubator. Baby rabbit complement and HL-60 cells ( $5 \times 10^5$ ) cultured 5 days in the presence of 90 mM DMF were added to the mixture and incubated at 37  $^{\circ}C$  for 1 hour under shaking. Aliquots were removed for quantitative culture. Titers were determined by extrapolating the antibody dilution corresponding to fifty percent live bacteria. The results of these

assays are shown in Table 5 for the pneumococcal type 14 conjugates and in Table 6 for the GBS type III conjugates.

*Serum bactericidal assay (SBA)* : Antibody-dependent complement-mediated bactericidal activity was measured in terms of the bactericidal titer, or reciprocal dilution, that provided 50% killing of the targeted bacteria. The complement in all sera was first incubated at 56 °C for 30 min. Then a 2-fold dilution series was established for each serum with GBSS in sterile 96-well U-bottom microtiter plates (Sigma), giving a final volume of 50 µL/ well. Infant rabbit serum complement (Pel-Freez, Brown Deer, WI) was diluted 1:1 with the working concentration of GBM bacteria (serotype 15 strain, 44/76) or Group C meningococcal C11 reference strain and 50 µL was added to each well containing the diluted serum, giving a final reaction mixture volume of 100 µL/ well. This reaction mixture, which contained 50% serum (heat-inactivated and diluted), 25% rabbit serum complement, and 25% bacteria (at working concentration), was incubated in a humidified incubator at 37 °C with 5% CO<sub>2</sub> for 60 min on a microtitration plate shaker (LKB-Wallac; pharmacia Biotech) at the fast speed.

All wells were then plated on chocolate Agar by spreading 30 µL/ plate. Time zero bacterial samples were also plated. All plates were incubated overnight, as before. The colony-forming units (cfu) were then counted with an automated colony counter from Imaging Products International (Chantilly, VA), taking an average of three readings per plate. The reciprocal dilution, or titer, that gave 50% killing was read directly from a graph constructed where the x-axis represented the log<sub>10</sub> value of the corresponding reciprocal dilution and the y-axis represented the percentage survival. The results of this assay are shown in Table 7.

**TABLE 5:**

**Immunogenicity of Pneumococcal 14-Tetanus Toxoid Conjugates**

Vaccine/ adjuvant	Elisa day0	Elisa day28	Elisa day38	Elisa day59	OP day59
Control / Saline	<50	2,250	29,000	32,600	3,100
Control / Alum	<50	18,200	99,000	265,000	25,000
Pn14-TT/ Saline	<50	4,600	89,000	59,200	3,900
Pn14-TT/ Alum	<50	27,000	185,000	251,000	26,000
PBS/ Alum	<50	<50	<50	<50	<50

Control vaccine was a type 14 polysaccharide-tetanus toxoid conjugate prepared by reductive amination. Pn14-TT conjugate was the product of direct coupling between an N-Acryloylated type 14 pneumococcal polysaccharide and tetanus toxoid.

For this study groups of 10 CD1 mice (Charles River Laboratory) aged 6-8 weeks, were injected subcutaneously with 2.0 µg of conjugated polysaccharide on days 0, 28 and 38. The animals were bled on days 0, 28, 38 and exsanguinated on day 59. ELISAs were performed using Pn14 polysaccharide-HSA conjugate prepared by reductive amination. The ELISA titers reported in Table 5 represent total IgGs. The reported OP titers are against pneumococcal type 14 strain.

**TABLE 6:**  
**Immunogenicity of GBS Type III Conjugates**

Vaccine/ Adjuvant	Elisa day 0	Elisa day 21	Elisa day 42	Elisa day 52	OP day 52
Control conjugate/ Alum	<50	900	1,800	3,000	170
GBS III-TT / Alum	<50	500	8,500	25,000	3,100
PBS / Alum	<20	<20	<20	<20	<20

Control conjugate vaccine was a GBS type III-Tetanus toxoid conjugate prepared by reductive amination of a periodate oxidized GBS type III polysaccharide and tetanus toxoid. GBS III-TT conjugate was the product of direct coupling between an N-Acryloylated type III polysaccharide and tetanus toxoid.

For this study groups of 10 CD1 mice (Charles River Laboratory) aged 6-8 weeks, were injected subcutaneously with 2 µg of conjugated polysaccharide on days 0, 21, and 42. Mice were bled on days 0, 21, 42, and exsanguinated on day 52. ELISA titers were measured using a GBS type III polysaccharide coupled to human serum albumin, titers given in Table 6 represent total IgGs to the type III polysaccharide.

**TABLE 7:**  
**Immunogenicity of E. Coli K1 Conjugates**

Vaccine / Adjuvant	Elisa day 0	Elisa day 28	Elisa day 42	Elisa day 52	SBA day 52
K1-rPorB II /Alum Control Lot1	<50	1,000	39,000	106,000	450
K1-rPorB II / Alum Control Lot 2	<50	450	124,000	250,000	1,000
K1-rPorB I/ Alum Lot 1	<50	220	27,000	96,000	810
K1-rPorB I/ Alum Lot 2	ND	ND	24,000	71,000	3,800
K1-S-rPorB/ Alum Lot 1	ND	ND	45,000	114,000	2,600
K1-S-rPorB /Alum Lot2	ND	ND	41,000	94,000	1,700
PBS / Alum	<50	<50	<50	<50	<50

5 Control vaccine (K1-rPorB II) was the product of reductive amination between a periodate-oxidized N-Acryloylated K1 polysaccharide and tetanus toxoid. K1-rPorB I vaccine was the product of direct coupling of an N-Acryloylated K1 polysaccharide and tetanus toxoid. K1-S-rPorB was the product of direct coupling of the thiolated porin rPorB and the N-Acryloylated K1 polysaccharide.

10 For these studies groups of 10 CD1 mice (4-6 weeks old) from Charles River laboratory were immunized intraperitoneally on days 0, 28, and 42. Mice were bled on days 0, 28, 42, and then exsanguinated on day 52. ELISAs titers were measured using an N-Propionylated K1 polysaccharide coupled to human serum albumin. Titers shown in Table 7 represent total IgGs to the modified N-Propionylated K1 polysaccharide. Serum bactericidal activities (SBA) against *N.meningitidis* group B serotype 15 H44/76 strain, for the day 52 bleed, are also shown in Table 7.

**TABLE 8:**  
**Immunogenicity of GCMP Conjugates**

<b>Vaccine/ Adjuvant</b>	<b>ELISA day 38</b>	<b>SBA day 38</b>
GCMP-S-rPorB/ Alum Lot1	44,000	2,100
GCMP-S-rPorB/ Alum Lot2	43,000	2,800
PBS/ Alum	<50	<50

- GCMP-S-rPorB was the product of direct coupling between the N-Acryloylated group C meningococcal polysaccharide (GCMP) and the thiolated rPorB. For these animal studies groups of 10 outbred Swiss Webster female mice (6-8 weeks old) from HSD were injected s.c. with 2 µg of conjugated polysaccharide per dose on days 0 and 28. Animals were exsanguinated on day 38. ELISA titers to the group C polysaccharide are measured using a GCMP coupled to human serum albumin. Serum bactericidal titers are obtained using the meningococcal C11 reference strain.

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- 5

We claim:

1. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate comprising an N-propionated polysaccharide or N-propionated oligosaccharide directly conjugated to a protein at the  $\beta$ -position of the propionate moiety.
2. A polysaccharide-protein conjugate according to claim 1 wherein the protein comprises at least one lysine or cysteine residue.
3. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from bacteria, yeast, cancer cells, or chemically synthesized.
4. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.
5. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide is derived from Group B streptococcus selected from the group consisting of serotype Ia, serotype Ib, serotype II, serotype III, serotype V, serotype VIII, and combinations thereof.
6. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 4 wherein the polysaccharide or oligosaccharide is derived from a Meningococcus group selected from the group consisting of group B, group C, group Y, group W135, and combinations thereof.
7. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 4 wherein the polysaccharide or oligosaccharide is derived from *E. coli* K1, *E. coli* K92, Pneumococcus type 4, Pneumococcus type 14, Streptococcus group A, Streptococcus group C, or combinations thereof.
8. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a *Neisseria meningitidis* outer membrane protein, pneumolysoid, C- $\beta$  protein from group B *Streptococcus* and non-IgA-binding C- $\beta$  protein from group B *Streptococcus*.

9. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 8 wherein the protein is recombinantly produced.

10. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 9 wherein the protein is recombinant *N. meningitidis* outer membrane protein.

11. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide comprises a glycosaminoglycan.

12. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the polysaccharide or oligosaccharide comprises glycosyl residues of a structural repeating unit having at least one free amino group or N-acyl group.

13. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 12 wherein the glycosyl residue is selected from the group consisting of glucosamine, galactosamine, mannosamine, fucosamine and sialic acid.

14. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 wherein the N-propionated polysaccharide or N-propionated oligosaccharide is directly conjugated to an  $\epsilon$ -free amino group of a lysine residue or a thiol group of a cysteine residue of the protein.

15. A polysaccharide-protein conjugate comprising N-propionated *Streptococcus pneumoniae* type 14 polysaccharide-tetanus toxoid conjugate, N-propionated Group B streptococcus type III polysaccharide-tetanus toxoid conjugate, N-propionated Group B Streptococcus type II polysaccharide-tetanus toxoid conjugate, N-propionated *E. coli* K1 polysaccharide-protein conjugate, or N-propionated meningococcal C polysaccharide-tetanus toxoid conjugate.

16. A polysaccharide-protein conjugate or oligosaccharide-protein conjugate produced by a method comprising:

A) de-N-acetylating an isolated polysaccharide or oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or a de-N-acetylated oligosaccharide,

B) N-acryloylating the de-N-acetylated polysaccharide or the de-N-acetylated oligosaccharide with an acryloylating reagent to form an N-propionated polysaccharide or an N-propionated oligosaccharide, and

5 C) directly conjugating the N-propionated polysaccharide or an N-propionated oligosaccharide to a protein to form the polysaccharide-protein conjugate or the oligosaccharide protein conjugate.

17. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 16 wherein the polysaccharide or oligosaccharide is derived from bacteria, yeast, cancer cells or chemical synthesis.

10 18. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted at a pH of about 7.0.

19. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted at a pH above 9.

15 20. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the conjugation is conducted in a reagent selected from the group consisting of phosphate, carbonate/bicarbonate buffer and borate buffer.

21. The polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 16 wherein the de-N-acetylating reagent is a base or an enzyme and the acryloylating reagent is selected from the group consisting of N-acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

22. A pharmaceutical composition comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claims 1 or 16 and  
25 a pharmaceutically acceptable carrier.

23. The pharmaceutical composition according to claim 22 further comprising an adjuvant.

24. The pharmaceutical composition according to claim 23 wherein the adjuvant is selected from the group consisting of alum or stearyl tyrosine.

30 25. The pharmaceutical composition according to claim 22 further comprising a second component, said second component selected from the group consisting of DTP, DTaP, Td, DTaP-Hib, DTaP-IPV-Hib, and combinations thereof.



26. A immunogen comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claims 1 or 16, said immunogen elicits a polysaccharide-specific or an oligosaccharide-specific immune response.

27. The immunogen according to claim 26, wherein the immune  
5 response is generation of polysaccharide-specific or an oligosaccharide-specific immunoglobulin.

28. The immunogen according to claim 27 wherein the immunoglobulin is IgG, IgM, IgA or combinations thereof.

29. A method of making a  $\beta$ -propionamido-linked polysaccharide-  
10 protein conjugate or a  $\beta$ -propionamido-linked oligosaccharide-protein conjugate comprising:

A) de-N-acetylating a polysaccharide or an oligosaccharide using a de-N-acetylating reagent to form a de-N-acetylated polysaccharide or de-N-acetylated oligosaccharide,

15 B) N-acryloylating the de-N-acetylated polysaccharide or de-N-acetylated oligosaccharide with an acryloylating reagent to form a  $\beta$ -propionated polysaccharide or a  $\beta$ -propionated oligosaccharide, and

C) directly conjugating the  $\beta$ -propionated polysaccharide or the  $\beta$ -propionamido oligosaccharide to a protein to form the  $\beta$ -propionamido-linked  
20 polysaccharide-protein or  $\beta$ -propionamido-linked oligosaccharide-protein conjugate conjugate.

30. The method of claim 29, wherein the de-N-acetylating reagent is a base or enzyme.

31. The method of claim 29 wherein the de-N-acetylating reagent is  
25 selected from the group consisting of NaOH, KOH and KiOH.

32. The method of claim 29, wherein the acryloylating reagent is selected from the group consisting of acryloyl chloride, acryloyl anhydride, acrylic acid and a dehydrating agent.

33. The method of claim 29, wherein the polysaccharide or  
30 oligosaccharide is derived from bacteria, yeast, cancer cells or chemical synthesis.

34. The method of claim 29 wherein the polysaccharide or oligosaccharide is derived from *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

35. The method of claim 29 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, a neisserial outer membrane protein, pneumolysoid, and C- $\beta$  protein from group B Streptococcus and non-IgA binding C- $\beta$  protein from group B Streptococcus.

36. The method of Claim 35, wherein the protein is recombinantly produced.

37. A vaccine comprising the polysaccharide-protein conjugate or oligosaccharide-protein conjugate according to claim 1 or 16, wherein said vaccine provides protective immunity against a disease causing organism or cell.

38. A vaccine according to claim 37 wherein the disease causing organism or cell is selected from the group consisting of bacteria, yeast, and cancer cell.

39. A vaccine according to claim 38 wherein the bacteria is *Escherichia coli*, Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

40. A vaccine according to claim 37 further comprising a second immunogen in combination with the polysaccharide-protein conjugate or oligosaccharide-protein conjugate said second immunogen selected from the group consisting of DTP, DTaP, Td, DTaP, Hib, DTaP-IPV-Hib and combinations thereof.

41. A method of immunizing a mammal against a disease causing organism or disease causing cell comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

42. A method of immunizing a mammal against *Streptococcus pneumoniae* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

43. A method of immunizing a mammal against Group B Streptococcus comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

44. A method of immunizing a mammal against Group B *Neisseria meningitidis* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

45. A method of immunizing a mammal against Group C *Neisseria meningitidis* comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

46. A method of immunizing a mammal against *Haemophilus influenzae* type B comprising administering to the mammal an immunizing amount of the vaccine according to claim 37.

47. A method of eliciting an antibody response to a polysaccharide or an oligosaccharide in a mammal comprising administering of an effective amount of the polysaccharide-protein conjugate or oligosaccharide-protein conjugate of claim 1 or 16.

48. An immunoglobulin or antigen-binding fragment thereof produced according to the method of claim 47.

49. The immunoglobulin according to claim 48, selected from the group consisting of IgG antibody, IgM antibody, IgA antibody and combinations thereof.

50. The immunoglobulin according to claim 49, wherein the antibody is an isolated IgG.

51. An isolated antibody or antigen binding fragment thereof elicited in response to the  $\beta$ -propionamido-linked polysaccharide-protein conjugate or  $\beta$ -propionamido-linked oligosaccharide-protein conjugate according to claim 1 and 16, said antibody or antigen fragment thereof specifically immunoreactive with N-propionated polysaccharide or N-propionated oligosaccharide and immunoreactive with a native N-acetylated polysaccharide from which the  $\beta$ -propionated polysaccharide or  $\beta$ -propionated oligosaccharide was derived.

52. The antibody or antigen binding fragment thereof according to claim 51 wherein the native N-acetylated polysaccharide is a component of bacteria, yeast or cancer cells.

53. The antibody or antigen binding fragment thereof according a claim 52 wherein the polysaccharide is derived from *Escherichia coli*,

Meningococcus, Pneumococcus, Streptococcus, Haemophilus, Neisseria, Salmonella, Klebsiella, or Pseudomonas.

54. The antibody or antigen binding fragment thereof according to claim 51 wherein the antibody is recombinantly produced.

5 55. A method of passive immunization against a disease causing organism or disease causing cells comprising administration of an effective amount of the immunoglobulin or antibody according to claim 48 or 51, said amount is sufficient to inhibit or kill the disease causing organism or disease causing cells.

56. The method of passive immunization according to claim 55  
10 wherein the immunoglobulin is an isolated IgG antibody or antigen binding fragment thereof.

57. The method of passive immunization according to claim 55 wherein the immunoglobulin is an isolated IgM antibody or antigen binding fragment thereof.

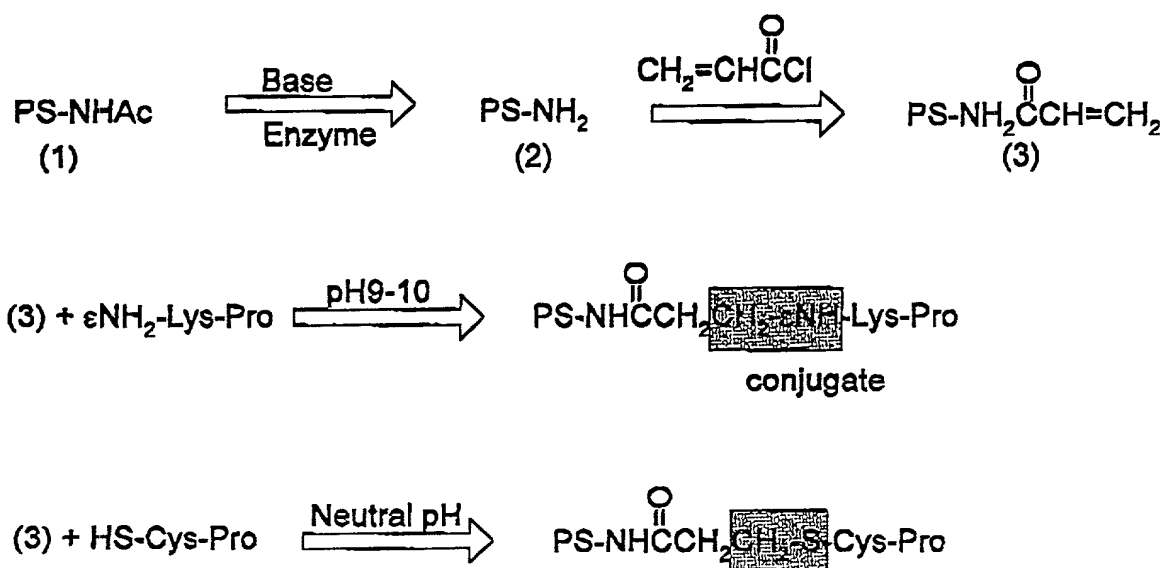
15 58. The method of passive immunization according to claim 55 wherein the immunoglobulin is an isolated IgA antibody or antigen binding fragment thereof.

### ABSTRACT

Novel immunogenic  $\beta$ -propionamido-linked polysaccharide- and N-propionamido-linked oligosaccharide-protein conjugates are provided as well as method of producing the conjugates. The conjugation procedure is simple, rapid, reproducible and applicable to a variety of polysaccharides or oligosaccharides derived from bacterial species, yeast, cancer cells or chemically synthesized. Vaccines and methods of immunization against infection or cancer using the immunogenic  $\beta$ -propionamido-linked polysaccharide- and  $\beta$ -propionamido-linked oligosaccharide-protein conjugates are also disclosed.

10

**FIGURE 1**



PS = Polysaccharide

Pro = Protein

COMBINED DECLARATION AND POWER OF ATTORNEY FOR  
PROVISIONAL, ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL  
DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION

As a below name inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

IMMUNOGENIC  $\beta$ -PROPIONAMIDO-LINKED POLYSACCHARIDE-PROTEIN CONJUGATE USEFUL AS A  
VACCINE PRODUCED USING AN N-ACRYLOYLATED POLYSACCHARIDE  
the specification of which

- a. ☒ is attached hereto
- b. ☐ was filed on \_\_\_\_\_ as application Serial No. \_\_\_\_\_ and was amended  
on \_\_\_\_\_. (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STATE

- c. ☐ was described and claimed in International Application No. \_\_\_\_\_ filed on \_\_\_\_\_  
and as amended on \_\_\_\_\_. (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby specify the following as the correspondence address to which all communications about this application are to be directed:

SEND CORRESPONDENCE TO: MORGAN & FINNEGAN, L.L.P.  
345 Park Avenue  
New York, N.Y. 10154

DIRECT TELEPHONE CALLS TO: KATHRYN M. BROWN  
(212) 758-4800

☐ I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or under § 365(b) of any foreign application(s) for patent or inventor's certificate or under § 365(a) of any PCT international application(s) designating at least one country other than the U.S. listed below and also have identified below such foreign application(s) for patent or inventor's certificate or such PCT international application(s) filed by me on the same subject matter having a filing date within twelve (12) months before that of the application on which priority is claimed:

☐ The attached 35 U.S.C. § 119 claim for priority for the application(s) listed below forms a part of this declaration.

<u>Country/PCT</u>	<u>Application Number</u>	<u>Date of filing (day, month, yr)</u>	<u>Date of Issue (day, month, yr)</u>	<u>Priority Claimed</u>
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO
				<input type="checkbox"/> YES <input type="checkbox"/> NO

☒ I hereby claim the benefit under 35 U.S.C. § 119(e) of any U.S. provisional application(s) listed below.

<u>Provisional Application No.</u>	<u>Date of Filing (day, month, yr)</u>
60/097,120	August 19, 1998

ADDITIONAL STATEMENTS FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART  
OR PCT INTERNATIONAL APPLICATION(S) (DESIGNATING THE U.S.)

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or under § 365(c) of any PCT international application(s) designating the U.S. listed below.

<u>US/PCT Application Serial No.</u>	<u>Filing Date</u>	<u>Status (patented, pending, abandoned)/ U.S. application no. assigned (For PCT)</u>

☐ In this continuation-in-part application, insofar as the subject matter of any of the claims of this application is not disclosed in the above listed prior United States or PCT international application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or Imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorneys and/or agents with full power of substitution and revocation, to prosecute this application, to receive the patent, and to transact all business in the Patent and Trademark Office connected therewith: John A. Diaz (Reg. No. 19,550), John C. Vassil (Reg. No. 19,098), Alfred P. Ewert (Reg. No. 19,887), David H. Pfeffer, P.C. (Reg. No. 19,825), Harry C. Marcus (Reg. No. 22,390), Robert E. Paulson (Reg. No. 21,046), Stephen R. Smith (Reg. No. 22,615), Kurt E. Richter (Reg. No. 24,052), J. Robert Dailey (Reg. No. 27,434), Eugene Moroz (Reg. No. 25,237), John F. Sweeney (Reg. No. 27,471), Arnold I. Rady (Reg. No. 26,601), Christopher A. Hughes (Reg. No. 26,914), William S. Feiler (Reg. No. 26,728), Joseph A.



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[ ] I hereby authorize the U.S. attorneys and/or agents named hereinabove to accept and follow instructions from \_\_\_\_\_ as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and/or agents and me. In the event of a change in the person(s) from whom instructions may be taken I will so notify the U.S. attorneys and/or agents hereinabove.

Full name of sole or first inventor Francis Michon

Inventor's signature\* Francis Michon July 29/1999  
date

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Full name of second joint inventor Chun-Hsien Huang

Inventor's signature\* [Signature] 29 July 99  
Date

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[X] ATTACHED IS ADDED PAGE TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR SIGNATURE BY THIRD AND SUBSEQUENT INVENTORS FORM.

\* Before signing this declaration, each person signing must:

1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

ADDED PAGE TO COMBINED DECLARATION  
AND POWER OF ATTORNEY FOR  
SIGNATURE BY THIRD AND SUBSEQUENT INVENTOR

Full name of third joint inventor, if any Catherine Uitz

Inventor's signature\*  8/5/99  
date

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Full name of fourth joint inventor, if any \_\_\_\_\_  
date

Inventor's signature\* \_\_\_\_\_

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Full name of fifth joint inventor, if any \_\_\_\_\_

Inventor's signature\* \_\_\_\_\_  
date

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Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

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1. Review the declaration and verify the correctness of all information therein; and
2. Review the specification and the claims, including any amendments made to the claims.

After the declaration is signed, the specification and claims are not to be altered.

To the inventor(s):

The following are cited in or pertinent to the declaration attached to the accompanying application:

Title 37, Code of Federal Regulation, § 1.56

Duty to disclose information material to patentability.

(a) A patent by its very nature is affect with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- and
- (1) prior art cited in search reports of a foreign patent office in a counterpart application,
  - (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

Title 35, U.S. Code § 101

Inventions patentable

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Title 35 U.S. Code § 102

Conditions for patentability; novelty and loss of right to patent

A person shall be entitled to a patent unless –

- (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for patent,
- (b) the invention was patented or described in a printed publication in this or foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States, or
- (c) he has abandoned the invention, or

(d) the invention was first patented or caused to be patented, or was the subject of an inventor's certificate, by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application for patent or inventor's certificate filed more than twelve months before the filing of the application in the United States, or

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent, or

(f) he did not himself invent the subject matter sought to be patented, or

(g) before the applicant's invention thereof the invention was made in this country by another had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other ...

#### Title 35, U.S. Code § 103

##### Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

#### Title 35, U.S. Code § 112 (in part)

##### Specification

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise and exact terms also enable any person skilled in the art to which it pertains, or with which it is mostly nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

#### Title 35, U.S. Code § 119

##### Benefit of earlier filing date in foreign country; right of priority

An application for patent for an invention filed in this country by any person who has, or whose legal representatives or assigns have, previously regularly filed an application for a patent for the same invention in a foreign country which affords similar privileges in the case of applications filed in the United States or to citizens of the United States, shall have the same effect as the same application would have if filed in this country on the date on which the application for patent for the same invention was first filed in such foreign country, if the application in this country is filed within twelve months from the earliest date on which such foreign application was filed; but no patent shall be granted on any application for patent for an invention which had been patented or described in a printed publication in any country more than one year before the date of the actual filing of the application in this country, or which had been in public use or on sale in this country more than one year prior to such filing.

Title 35, U.S. Code § 120

Benefit or earlier filing date in the United States

An application for patent for an invention disclosed in the manner provided by the first paragraph of section 112 of this title in an application previously filed in the United States, or as provided by section 363 of this title, which is filed by an inventor or inventors named in the previously filed application shall have the same effect, as to such invention, as though filed on the date of the prior application, if filed before the patenting or abandonment of or termination of proceedings on the first application or an application similarly entitled to the benefit of the filing date of the first application and if it contains or is amended to contain a specific reference to the earlier filed application.

Please read carefully before signing the Declaration attached to the accompanying Application.

If you have any questions, please contact Morgan & Finnegan, L.L.P.

FORM:COMB-DEC.NY  
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